


PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P200300646WO		FOR FURTHER ACTION		See Form PCT/IPEA/416
International application No. PCT/DK2004/000478		International filing date (day/month/year) 02.07.2004		Priority date (day/month/year) 03.07.2003
International Patent Classification (IPC) or national classification and IPC C12N9/12, C12N15/54, C12N15/82, A01H5/00, C12Q1/68				
Applicant AARHUS UNIVERSITET et al.				
<p>1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 6 sheets, including this cover sheet.</p> <p>3. This report is also accompanied by ANNEXES, comprising:</p> <p>a. <input checked="" type="checkbox"/> sent to the applicant and to the International Bureau) a total of 5 sheets, as follows:</p> <p><input checked="" type="checkbox"/> sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).</p> <p><input checked="" type="checkbox"/> sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.</p> <p>b. <input type="checkbox"/> (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).</p>				
<p>4. This report contains indications relating to the following items:</p> <p><input checked="" type="checkbox"/> Box No. I Basis of the opinion</p> <p><input checked="" type="checkbox"/> Box No. II Priority</p> <p><input type="checkbox"/> Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p><input type="checkbox"/> Box No. IV Lack of unity of invention</p> <p><input checked="" type="checkbox"/> Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p><input type="checkbox"/> Box No. VI Certain documents cited</p> <p><input type="checkbox"/> Box No. VII Certain defects in the international application</p> <p><input type="checkbox"/> Box No. VIII Certain observations on the international application</p>				
Date of submission of the demand 03.02.2005		Date of completion of this report 21.10.2005		
Name and mailing address of the international preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016		Authorized Officer Blanco Urgoiti, B Telephone No. +31 70 340-4148		



**INTERNATIONAL PRELIMINARY REPORT
ON PATENTABILITY**

International application No.
PCT/DK2004/000478

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.
- ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
- ☐ international search (under Rules 12.3 and 23.1(b))
 - ☐ publication of the international application (under Rule 12.4)
 - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)
2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-68 as originally filed

Sequence listings part of the description, Pages

1-61 as originally filed

Claims, Numbers

1-32 filed with the demand

Drawings, Sheets

1/13-13/13 as originally filed

- ☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing
3. ☒ The amendments have resulted in the cancellation of:
- ☐ the description, pages
 - ☒ the claims, Nos. 1-26
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):
4. ☒ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
- ☐ the description, pages
 - ☒ the claims, Nos. 26,28
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):

* If item 4 applies, some or all of these sheets may be marked "superseded."

**INTERNATIONAL PRELIMINARY REPORT
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Box No. II Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
- ☐ copy of the earlier application whose priority has been claimed (Rule 66.7(a)).
 - ☐ translation of the earlier application whose priority has been claimed (Rule 66.7(b)).
2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rule 64.1). Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.
3. Additional observations, if necessary:
- see separate sheet**

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	1-25,27,29-32
	No: Claims	
Inventive step (IS)	Yes: Claims	1-25,27,29-32
	No: Claims	
Industrial applicability (IA)	Yes: Claims	1-25,27,29-32
	No: Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

**INTERNATIONAL PRELIMINARY REPORT
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Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this report has been established on the basis of:
 - a. type of material:
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☒ in written format
 - ☒ in computer readable form
 - c. time of filing/furnishing:
 - ☒ contained in the international application as filed
 - ☒ filed together with the international application in computer readable form
 - ☐ furnished subsequently to this Authority for the purposes of search and/or examination
 - ☐ received by this Authority as an amendment on
2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional observations, if necessary:

Re Item I.

The amendments filed with the International Bureau under Article 19(1) introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 19(2) PCT. The amendments concerned are the following:

- No basis in the application as filed can be found for the transgenic monocot plant of claim 26, in general. The change of "cereal" for "monocotyledonous plant" in general makes the scope of the claim broader and contrary to Article 19(2) PCT.

-The description as filed does not disclose that the term "substantially similar" is equivalent to "at least 60% identical to". Page 19, lines 9-10 of the application as filed refers to the term "substantially identical".

Re Item II.

The priority date claimed is valid for all claims presently on file. However, if at a later date this appears not to be the case, the documents cited as "P" in the International Search Report will become relevant for assessing novelty and inventive step (Art. 33 (1)-(3) PCT).

Re Item V.

1 The following documents are referred to in this communication:

D3: SCHAUSER L ET AL: "Symbiotic mutants deficient in nodule establishment identified after T-DNA transformation of *Lotus japonicus*" MOLECULAR AND GENERAL GENETICS, vol. 259, no. 4, September 1998, pages 414-423.

2. NOVELTY AND INVENTIVE STEP (Arts. 33(2) and (3) PCT)

2.1 D3 is considered to be the closest prior art. It discloses a genetic characterization of mutants of *Lotus japonicus* which are unable to establish symbiosis with *Mesorhizobium loti*. Among them *sym1* and *sym5* mutants are deficient in developing

the nodules but able of establishing mycorrhiza.

- 2.2 In view of the prior art, the subject-matter of claims 1 to 25, 27, and 29 to 32 is novel (Art. 33(2) PCT)
- 2.3 In view of D3, the problem to be solved by the present application is the cloning and sequencing of the genes responsible for the *sym1* and *sym5* mutants. The solution is the provision of two LysM receptor-like kinases (NFR1 and NFR5) from various legumes, their functional characterization and the different applications as claimed.
- 2.4 As it is not considered to be obvious to arrive to the proteins of the invention, and moreover, to the finding that they interact together in the reception of the nod-factors, being the determinants of host range in the legume roots, an inventive step can be acknowledged to the subject-matter of claims 1 to 25, 27, and 29 to 32

3. ARTICLE 6 PCT

- 3.1 The term "NFR" used in claims 18, 29, 30 has no well-recognised meaning and leave the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).
- 3.2 The term "a **specific** Nod-factor binding **property**" used in claim 1 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT). It is not clear if the term "specific" refers to taxonomical species in general, or to a rhizobial strain specificity as disclose all through the description.

4. INDUSTRIAL APPLICABILITY

- 4.1 The subject-matter of claims 1 to 25, 27, and 29 to 32 seems to be susceptible of industrial applicability as defined in Article 33(4) PCT.

International Application No: PCT/DK2004/000478

Amended Claims (filed 10. 02.2005)

- 5 1. An isolated Nod-factor binding element comprising one or more isolated Nod-factor binding polypeptide (NFR polypeptide) having a specific Nod-factor binding property, or a functional fragment thereof, wherein the amino acid sequence of said NFR polypeptide is at least 60% identical to any one of SEQ ID NO: 8, 15 or 25.
- 10 2. The Nod-factor binding element of claim 1, wherein said NFR polypeptide is NFR1, comprising the amino acid sequence selected from the group consisting of SEQ ID No: 24, 25, 52 and 54.
- 15 3. The Nod-factor binding element of claim 1, wherein the NFR polypeptide is NFR5 comprising an amino acid sequence selected from the group consisting of SEQ ID No: 8, 15, 32, 40 and 48.
- 20 4. The Nod-factor binding element of claim 1, comprising
 - a. a NFR polypeptide, or a functional fragment thereof, wherein the amino acid sequence of said NFR polypeptide is at least 60% identical to SEQ ID No: 24 or 25; and
 - b. a NFR polypeptide or a functional fragment thereof, wherein the amino acid sequence of said NFR polypeptide is at least 60% identical to a sequence selected from the group consisting of SEQ ID No: 8, 15, and 32.
- 25 5. The Nod-factor binding element of claim 1, comprising:
 - a. the NFR polypeptide that is NFR1 or a functional fragment thereof, having the amino acid sequence selected from the group consisting of SEQ ID No: 24, 25, 52 and 54, and
- 30

b. the NFR polypeptide that is NFR5 or a functional fragment thereof, having an amino acid sequence selected from the group consisting of SEQ ID No: 8, 15, 32, 40 and 48.

- 5 6. An isolated nucleic acid molecule encoding a NFR polypeptide according to claim 1, wherein the NFR amino acid sequence is at least 60% identical to either of SEQ ID NO: 8, 15 or 25.
- 10 7. An isolated nucleic acid molecule encoding a NFR 1 polypeptide according to claim 2, comprising the amino acid sequence selected from the group consisting of SEQ ID No: 24, 25, 52 and 54.
- 15 8. An isolated nucleic acid molecule encoding a NFR 5 polypeptide according to claim 3, comprising an amino acid sequence selected from the group consisting of SEQ ID No: 8, 15, 32, 40 and 48.
- 20 9. An isolated nucleic acid molecule which encodes a Nod-factor binding polypeptide of a Nod-factor binding element, wherein said polypeptide is NFR1, and wherein said nucleic acid molecule hybridises with a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID No: 21, 22, 23, 51 and 53 under stringency conditions of no less than about 1.0xSSC at 65°C.
- 25 10. An isolated nucleic acid molecule which encodes a Nod-factor binding polypeptide of a Nod-factor binding element, wherein said polypeptide is NFR5, and wherein said nucleic acid molecule hybridises with a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID No: 6, 7, 11, 12, 39 and 47 under stringency conditions of no less than about 1.0xSSC at 65°C.

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11. An expression cassette comprising a nucleic acid molecule according to any one of claims 6 to 10.
- 5 12. An expression cassette comprising a nucleic acid molecule according to claim 6.
13. The expression cassette of claim 11, wherein the nucleic acid molecule encoding a NFR polypeptide is operably linked to a transcriptional regulatory element.
- 10 14. A vector comprising the expression cassette of claim 13.
15. A cell that is stably transformed with the expression cassette of claim 13.
- 15 16. The cell according to claim 15, wherein said cell is a plant cell.
17. A method of producing a plant expressing a Nod-factor binding element, the method comprising introducing into the plant a transgenic expression cassette comprising a nucleic acid sequence encoding a NFR polypeptide according to any one of claims 5 to 9, wherein the nucleic acid sequence is operably linked to a promoter and selecting transgenic plants and their progeny expressing said NFR polypeptide.
- 20 18. A method of producing a plant expressing a Nod-factor binding element, the method comprising introducing into the plant a transgenic expression cassette comprising a nucleic acid sequence encoding a NFR polypeptide, wherein the amino acid sequence of said NFR polypeptide is at least 60% identical to any one sequence selected from the group consisting of SEQ ID NO: 8, 15, 25 and 32.
- 25 30

19. The method of claim 17 or 18, wherein the transgenic expression cassette is introduced into the plant through a sexual cross.
- 5 20. The method of claim 17 or 18, wherein said promoter is a native or heterologous root specific promoter.
21. The method of claim 17 or 18, wherein said promoter is a native or heterologous constitutive promoter.
- 10 22. A transgenic plant expressing one or more NFR polypeptides produced according to the method of any one of claims 17 to 21.
23. A transgenic plant expressing one or more NFR polypeptides, produced according to the method of claim 18.
- 15 24. The transgenic plant of claim 22 or 23, expressing the Nod-factor binding element according to any one of claims 1 to 4, and having a specific rhizobial strain recognition.
- 20 25. The transgenic plant of any one of claims 22 to 24, wherein the plant is a non-nodulating dicotyledenous plant.
26. The transgenic plant of any one of claims 22 to 24, wherein the plant is a monocotyledonous plant.
- 25 27. The transgenic plant of claim 26, wherein said monocotyledonous plant is a cereal.
- 30 28. A method for marker assisted breeding of *NFR* alleles, encoding variant Nod-factor binding polypeptides (*NFR* polypeptides), comprising the steps of:

- 5
- a. determining the nodulation frequency of legume plants expressing a variant NFR polypeptide having specific Nod-factor binding properties and having an amino acid sequence at least 60% identical to a sequence selected from the group consisting of SEQ ID No: 8, 15, 24, 25, 32, and
- b. identifying a DNA polymorphism at a locus genetically linked to or within the allele encoding said variant NFR polypeptide, and
- c. preparing a molecular marker based on said DNA polymorphism, and
- 10 d. using said molecular marker for the identification and selection of a plant carrying an *NFR* allele encoding said variant NFR polypeptide.
- 15 29. The method according to claim 27, wherein said variant NFR polypeptide has an amino acid sequence substantially similar to a sequence selected from the group consisting of SEQ ID No: 8, 15, 24, 25, 32, 40, 48, 52 and 54.
- 20 30. A plant selected according the method of claim 27 or 28, carrying a *NFR* allele encoding a variant NFR polypeptide.
- 25 31. Use of the method of claim 27 or 28 for breeding a plant with enhanced nodulation frequency and/or root nodule occupancy and/or enhanced symbiotic nitrogen fixation ability.
32. A use according to claim 30, wherein said plant is a legume.